AD_____

Award Number: W81XWH-10-2-0110

TITLE: Microvesicle Production After Trauma and Its Clinical Impact on Venothromboembolism

PRINCIPAL INVESTIGATOR: Dr. Myung Park

CONTRACTING ORGANIZATION: Mayo Clinic
Rochester, MN 55905

REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2011	Annual	27 Sep 2010 - 26 Sep 2011

4. TITLE AND SUBTITLE

Microvesicle Production After Trauma and Its Clinical Impact on Venothromboembolism

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-10-2-0110

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Myung Park

E-Mail: park.myung@mayo.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mayo Clinic
Rochester, MN 55905

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Polytrauma is most often caused from explosive devices and accounts for about 65% of injuries to our military personnel. People with polytrauma are at increased risk of developing either bleeding and/or a clot in their veins which cause a life-threatening event known as venous thromboembolism (VTE). We began enrollment of patients into the study on 2 February, 2011. As of 21 September, 2011, we have successfully enrolled and collected blood samples on 279 patients and 64 healthy volunteers. We also began analyzing plasma samples to assess their potential for thrombin generation.

15. SUBJECT TERMS

Polytrauma, coagulopathy, venous thromboembolism

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	31	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction: Venous thromboembolism (VTE) is one of seven adverse events faced by combat casualties reported monthly to the Department of Defense. Rates of symptomatic and asymptomatic deep vein thrombosis (DVT) and pulmonary embolism (PE) in trauma patients are as great as 44 and 24%, respectively. Currently, the standard guideline is that all major trauma patients receive VTE chemoprophylaxis. This practice exposes those not at risk for thrombosis to potentially serious or even fatal bleeding and there are no adequate laboratory tests currently available to target anticoagulant prophylaxis to those that need it most. The **central hypothesis** of this proposal is that traumatic injury results in the release of procoagulant and pro-inflammatory factors found both in plasma and microvesicles (MVs) derived from blood cells and injured tissues. The specific Aims of this study are:

Aim #1:

- Identify cellular origins and quantitate procoagulant microvesicles (MVs) defined by cell specific markers in patients with acute traumatic injury.
- Determine the basis of differences in native and tissue-factor stimulated thrombin generation.

Aim #2:

- Develop a predictive signature for a pre-thrombotic individual: thrombin generation concurrent with thrombogenic microvesicles.

Body: Upon receipt of the award in November 2010, we received approval to launch the study in mid-January 2011:

Task/Milestone #1 - Project launch and planning:

- The primary investigator (PI) met with the co-investigators, research coordinators, research laboratory technician and staff from the Clinical Research Unit (CRU) of the Center for Translational Science Activities (CTSA) to set timeline of duties, work expectations and plan of action needed to achieve the first milestone. Milestone #1 was reached on February 2, 2011 with a launch of the study by meeting with all of the co-investigators to (1) review project expectations; (2) review scheduling and deliverables; and (3) prioritize available resources.

- The primary investigator gave multiple presentations and information sessions in various wards and intensive care units at Saint Mary's Hospital where trauma patients are managed. Questions concerning the study protocol by nursing and physician staff were addressed by the primary investigator.
- We also identified and trained all study coordinators and participants in the process and logistics of execution of the study protocol. We also finalized the system of procedure (SOP) of blood draws to be performed by the Clinical Research Unit (CRU) within the Center for Translational Science Activities (CTSA) at Mayo Clinic, Rochester, Minnesota.

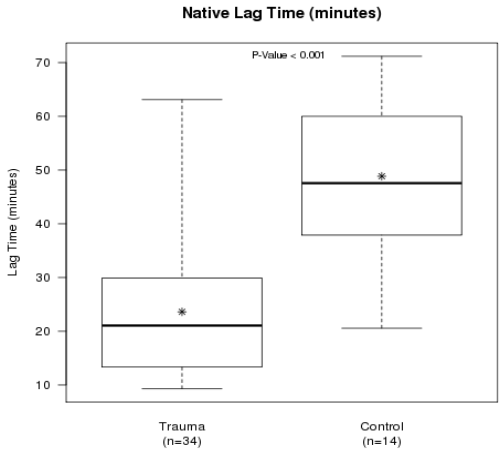
Task/Milestone #2

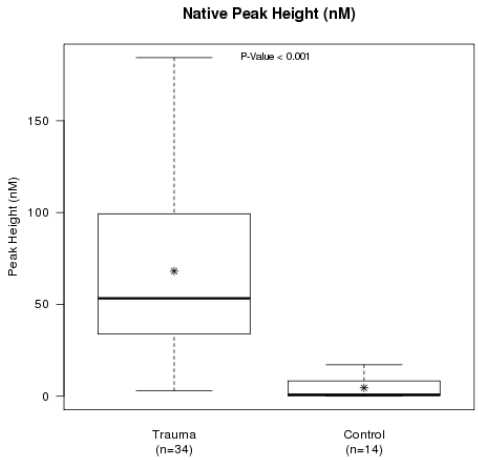
- As of 21 September 2011, we have enrolled and obtained consents from 279 patients and 64 healthy volunteers into the proposed study. The total number of subjects who had their blood drawn per study protocol is 541 (472 trauma patients and 66 controls). Of the 66 controls, two controls met exclusion criteria (open screen failures). Of the 472 patients who had their blood drawn per protocol by the Clinical Research Unit (CRU), 85 patients met exclusion criteria, 37 patients declined to participate, 41 patients were mailed a cover letter and consent forms that were never returned, and 30 patients were recently mailed a cover letter and consent form which are pending return. We had one patient withdraw consent after he was discharged from the hospital. There have been no screened failures to date.

- The initial 4.5 months of the study was focused on streamlining and optimizing the clinical-end of the research project; this entailed coordination at multiple levels to obtain blood draw from trauma patients relative to their time of injury. In regards to the laboratory assessment of the plasma samples, the first 4.5 months were spent on standardizing our methods to perform the thrombin generation assay by calibrated automated thrombinogram (CAT) and MV analysis by flow cytometry. This entailed the use of reference plasma to obtain consistent and reproducible results using CAT by the investigators and

methods (1).

- This is a preliminary analysis of selected plasma samples that were analyzed. The plasma samples obtained within the first 6 hours of injury are represented here. The data are presented as Mean ± SD unless otherwise noted. P value of < 0.05 was considered to be statistically significant:
    - Of the 38 patients analyzed, 68% were men. The mean injury severity score was 17.3 ± 13.5, time since injury to admission 4.8 ± 3.6 hours, Systolic BP 109 ± 19 mm Hg, hospital length of stay 7.8 ± 8.8 days. Three of these patients developed venous thromboembolism.
    - The CAT was performed with and without exogenous agonists (tissue factor/ phospholipid). The performance of CAT without agonists, so called, native CAT (nCAT) revealed a shortened native Lag Time ($p < 0.001$) and increased native Peak Height ($P < 0.001$) as compared to the healthy control.
    - The rationale for performing native CAT was to be able to detect all procoagulant activity, particularly tissue factor (TF) generated after traumatic injury that might be otherwise masked by addition of high concentrations of exogenous agonists.
    - The CAT performed in a standard fashion (with agonists) did not differ significantly from the controls (data not shown).





- The MV analysis was performed on 22 trauma patients. The table below illustrates the types of MV evaluated:

Types of Microvesicles (MV)	Mean ± SD (# per uL plasma)
Annexin V binding Positive (thrombogenic)	92.1 ± 101.7
Platelet Derived (CD42a positive)	35.5 ± 39.3
Endothelial Derived (CD62E positive)	15 ± 40
Smooth Muscle Derived (SM)	4.8 ± 8

- Spearman rank correlations between the MV data and CAT data were performed. When correlations between MV and nCAT were performed, the correlation coefficients were significant with smooth muscle derived MV only:

	nCAT variable	r
Smooth Muscle MV	Lag Time (minutes)	- 0.62 (p = 0.002)
Smooth Muscle MV	Peak Height (nM)	0.52 (p = 0.01)

- Hence, our preliminary analysis indicates that trauma patients have an acceleration of thrombin generation (shortened Lag Time) and increased thrombin generation (greater Peak Height). Additionally, there appears to be correlation of smooth muscle derived MV and Lag Time and Peak Height. However,

- Microvesicle analysis utilizing flow cytometry is a relatively labor-intensive procedure and while the flow cytometry will be used throughout this study, other laboratory methods to assess for the presence of thrombogenic microvesicles in plasma will need to be pursued. Development of capture assays to assess the amount of Annexin V binding microvesicle in plasma would be an important avenue to pursue for future research projects. Overall, the CAT analysis is a superlative functional assay and the future direction of will entail using different levels of agonists (tissue factor and phospholipid) to delineate subtle thrombin generation differences amongst trauma patients with varied degree of injury.

Key Research Accomplishments:

- Launch of a novel trauma study to investigate the potential predictive value of thrombogenic microvesicles and thrombin generation characteristics to predict venous thromboembolism.
- Multi-departmental coordination to carry out and launch the prospective observational case-cohort study.
- Standardization of methods to perform the thrombin generation by calibrated automated thrombinogram (CAT) and MV analysis by flow cytometry.
- Training of the laboratory technical staff to yield reproducible and consistent results using reference-controlled plasma during the performance of the CAT and MV analysis on flow cytometry.
- Plasma sample analysis of trauma patients and healthy volunteers begun.

Reportable Outcomes:

As the study is still early in its inception, we do not have manuscripts, abstracts or presentations that have directly resulted from the research.

Conclusion:

It is the objective of this protocol to elucidate the role of microvesicles derived from cells and injured tissues as a source of thrombin generation after trauma. At the conclusion of our completed research, we should be able to identify the pattern of cellular origins and quantitate procoagulant microvesicles defined by cell specific markers in patients with acute traumatic injury. As outlined in AIM 2, it is the goal of our study to develop a VTE predictive model for an individual who, based on the thrombin generation and microvesicle analysis, can be stratified into low versus high risk for deep vein thrombosis and pulmonary embolism after trauma. In essence, the outcome of this research can help shape the future prospective multicenter research to validate our VTE predictive model and, in thus doing, allow clinicians to stratify patients into high versus low risk for post-injury venous thromboembolism. This is a novel study in its scale and scope.

Currently, flow cytometry is considered the standard instrument used for microvesicle analysis. However, flow cytometers are limited in their ability to detect particles less than 300 nanometers. It is imperative that we incorporate into our future research other devices designed to detect microvesicles smaller than 300 nanometers. The use of so called nanoparticle tracking devices are available. However, there are currently no fluorogenic antibodies that are compatible with these devices and this has been an impediment for wider use of nanotechnology in MV research. It will be important to keep “in-tune” to this evolving nanotechnology.

References:

1. Park MS, Owen BAL, Ballinger BA, Sarr MG, Schiller HJ, Jenkins DH, Zietlow SP, Ereth MH, Owen WH, Heit JA: Quantification of hypercoagulable state after bunt trauma: Microparticle and thrombin generation are increased relative to injury severity, while standard markers are not. Surgery (accepted for publication).

Appendices: (Reference article #1 for the methods used to perform the CAT and MV analysis)

Title: Quantification of Hypercoagulable State After Blunt Trauma: Microparticle and Thrombin Generation Are Increased Relative to Injury Severity, While Standard Markers are Not

Short Title: Assessing Hypercoagulable State after Trauma

Authors: Myung S. Park, M. D.[1], Barbara A. L. Owen, Ph. D.[1,2], Beth A. Ballinger, M. D.[1], Michael G. Sarr, M. D.[1], Henry J. Schiller, M. D.[1], Donald H. Jenkins, M. D.[1], Scott P. Zietlow, M. D.[1], Mark H. Ereth, M. D.[5], Whyte G. Owen, Ph. D.[2,6], John A. Heit, M. D.[2,3,4]

Department of Surgery[1], Hematology Research[2],Cardiovascular Diseases[3],Laboratory Medicine and Pathology[4], Anesthesiology[5], and Biochemistry and Molecular Biology[6], Mayo Clinic, Rochester, MN

Corresponding Author: Myung S. Park, M.D., Mayo Clinic, 200 First Street SW, Rochester, MN 55905; (507) 255-2923/ (507) 255-9872 (fax); park.myung@mayo.edu

Podium Presentation: This paper was the subject of oral presentation at the 40th Annual Western Trauma Association, Telluride, Colorado, 2010.

Disclosure of Funding: The project described was supported by Grant Number 1 K08 GM093133-01 from the National Institute of General Medical Sciences (NIGMS) and 1 UL1 RR024150 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and the NIH Roadmap for Medical Research . Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on NCRR is available at http://www.ncrr.nih.gov/

Objective: Major trauma is an independent risk factor for developing venous thromboembolism (VTE). While increases in thrombin generation and/or procoagulant microparticles (MP), have been detected in other patient groups at higher risk for VTE, such as cancer or coronary artery disease, this association has yet to be documented in trauma patients. This pilot study was designed to characterize and quantify thrombin generation and plasma MP in individuals early after traumatic injury.

Methods: Blood was collected in the trauma bay from 52 blunt injured patients (case) and 19 non-injured outpatients (controls) and processed to platelet poor plasma for 1) isolation of MP for identification and quantification by flow cytometry; and 2) in vitro thrombin generation as measured by calibrated automatic thrombography (CAT). Data collected are expressed as either mean ± standard deviation or median with interquartile range.

Results: Among cases, 39 men and 13 women (age = 40 ± 17), the injury severity score was 13 ± 11, INR 1.0 ± 0.1, PTT 25 ± 3 (sec), and platelet count 238 ± 62 (thousands). The numbers of total (cell-type not specified) procoagulant MP, as measured by Annexin V staining, were increased compared to non-trauma controls (541 ± 139/μl and 155 ± 148/μl, respectively, $p<0.001$). There was no significant difference in the amount of thrombin generated in trauma patients compared to controls; however, peak thrombin was correlated to injury severity, (Spearman correlation coefficient $R= 0.35$, $p=0.02$).

Conclusion: Patients with blunt trauma have greater numbers of circulating procoagulant MP and increased *in vitro* thrombin generation. Future studies, to characterize the cell-specific profiles of MP and changes in thrombin generation kinetics post-traumatic injury will determine whether they contribute to the hypercoagulable state observed after injury.

Key Words

1. An V+ Annexin V binding positive
2. CAT Calibrated Automated Thrombogram
3. LT Lag Time (minutes)
4. MP microparticles
5. PH Peak Height (nM)

INTRODUCTION:

Trauma Induced Coagulopathy (TIC) detected early after injury reflects injury severity and is prognostic for blood transfusions and death.[1-2] Maintenance of hemostasis, often with blood products aimed at limiting hemorrhage, comes with a price of increased risk of venous thromboembolism (VTE).[3-5] Although major trauma is an independent risk factor for VTE, injury in itself is not predictive of VTE for an individual patient; indeed, most trauma patients do not develop symptomatic VTE. The two faces of TIC, the hypo- and the hypercoagulable states, are poorly understood. Standard coagulation tests, which include prothrombin time (PT) and activated thromboplastin time (aPTT) assays, are measurements of the canonical extrinsic and intrinsic clotting pathways. They utilize excess concentrations of tissue factor or contact activator, which makes these tests relatively insensitive to perturbation of the clotting cascade because the end-points of these assays coincide with the onset of the propagation phase of clotting where >95% of thrombin is still to be generated.[6-7]

Clearly, tools having enhanced sensitivity and specificity are needed to understand the basis for TIC, to explore its relation to VTE, and to target risky, anticoagulant-based prophylaxis to those patients who would benefit most. To this end, two unconventional methods to assess hyper-coagulation were employed in this study. First is the Calibrated Automated Thrombogram (CAT), a plate-based assay that measures the rate of thrombin generation and inhibition in citrated plasma, and has been used to quantify procoagulant activity in several diseases including VTE and coronary artery disease.[8-10] The clinical utility of in vitro thrombin generation however, remains to be determined in trauma patients.

Second, circulating procoagulant microparticles (MP) have emerged as possible contributors to, or markers for, the hypercoagulable state. These sealed membrane vesicles arise from cell stress and other metabolic perturbations, and are shed into circulation from platelets, leukocytes, and endothelial cells. Increases in

circulating MP have been associated with inflammatory and thrombotic processes and represent an opportune analyte owing to facility of access to peripheral blood samples and available technology, such as flow cytometry, can identify both the procoagulant nature and the cell source of MP in plasma samples.

The objective of this study was to characterize and quantify procoagulant activity and MP in plasma obtained from individuals after traumatic injury.

PATIENTS AND METHODS

Study Design:

The study was approved by the Mayo Clinic IRB (# 09-000713) and was conducted in compliance with the Health Insurance Portability and Accountability Act (HIPPA) Regulations.

A prospective cohort study included consecutive patients from southeastern Minnesota, northern Iowa, or western Wisconsin arriving at the Mayo Clinic Trauma Center after suffering from blunt (including closed head injury) or penetrating injury. The patients were transported to the Mayo Clinic Emergency Department (ED) by ambulance or helicopter. Exclusion criteria were: age < 18 years, active treatment for anticoagulation (e.g., heparin, warfarin) or antithrombotic therapy (e.g., thienopyridine; excluding aspirin or non-steroidal anti inflammatory drugs), preexisting coagulopathy, more than 12 hours from time of injury, any transfusion of blood products prior to blood sample collection, active malignancy, sepsis or renal failure, or burn injuries. The clinical practice of the Trauma Service does not involve routine DVT surveillance by any form of routine, serial, noninvasive imaging in asymptomatic patients either during or after hospital discharge, in accordance with the guidelines of the American College of Chest Physicians on prophylaxis for VTE.[11] Clinical data were collected and included: injury severity score (ISS), Glasgow Coma Scale (GCS), body temperature, lactate, cell

blood count (CBC), activated partial thromboplastin times (aPTT) and international normalized ratio (INR). These particular parameters were collected as they have been shown to be affected by traumatic injury. [12-16]

Outpatients with no prior history of thrombosis (i.e., stroke, myocardial infarction or venous thromboembolism) seen at the Mayo Clinic during the same time period, provided blood samples for MP and CAT (n=19) reference (control) analyses. The individuals were not receiving anticoagulation (heparin, warfarin) or taking antithrombotics (e.g., thienopyridine; including aspirin or non-steroidal anti inflammatory drugs) within 7 days prior to blood sample collection.

Sample Collection and Processing:

To minimize the impact of confounding therapy, a one-time blood sample was collected either at the scene of the injury or, if the pre-hospital team was unable to collect the sample, in the ED. Whenever possible, written informed consent was obtained from the patient prior to collection of blood sample. When patients were unable to provide consent at the time of the trauma, consent was obtained from the patient or legal guardian prior to patient discharge; otherwise, the study sample was appropriately discarded. The study was approved by the Mayo Clinic Institutional Review Board.

Blood samples:

A total of 18 mL of blood was collected by antecubital venipuncture into 1) an anticoagulant containing hirudin (inhibits thrombin) plus soybean trypsin inhibitor (inhibits factor Xa) for the MP analysis 2) a 3.2% citrate tube for CAT analysis. Multiple aliquots of platelet poor plasma (PPP) were prepared by two (2) centrifugations (3000g, 15 minutes) and then frozen at -80 ° C until analysis.

CAT assay:

Procoagulant activity of plasma samples to which 50 ug/mL corn trypsin inhibitor (CTI, Haematologic Technologies Inc. Essex Junction, VT) had been added were assayed with the Calibrated Automated Thrombogram (CAT) as described by Hemker et al[17-18] and Owen et al. [19] For the canonical CAT, 5 nM exogenous tissue factor (TF) and 4 nM phospholipids (PL) was added to trigger thrombin generation. The CAT assay can be configured without addition of exogenous triggers, and is referred to as native CAT (nCAT).[19-20] The rationale for using native CAT was to be able to detect all procoagulant activity, particularly tissue factor (TF) generated after traumatic injury that might be otherwise masked by addition of high concentrations of exogenous activators.

MP Analysis:

Isolation and characterization of MP was carried out as described by Jayachandran et al.[19] Briefly, MP were collected by high speed centrifugation (20,000g for 30 min) and then stained with a platelet-specific fluorescent antibody (anti-CD42) and with fluorescent annexin V, which binds to procoagulant phosphatidylserine. The stained MP were counted with a FACSCanto flow cytometer (BD Biosciences, San Jose CA), with use of an internal standard of microbeads.

Statistical Analyses:

Data analysis was performed using SigmaStat 3.50 (Systat Software, Point Richmond, CA, USA). Student's t-test and the Mann-Whitney Rank Sum Test (as appropriate) were used to assess differences in mean values between the enrolled subjects and controls. Correlation was assessed with Spearman rank correlation analysis using Sigmaplot 8 (Systat Software, Inc. San Jose, CA). Data are expressed either as mean ± standard deviation (SD) or as median values with interquartile range; a $p < 0.05$ considered to be significant.

RESULTS:

Patient and Normal Donor Characteristics:

During a 6-week period, 6 April through 24 May 2009, one blood sample was collected from 80 of 147 patients admitted with major trauma (57 Level 1, based on physiologic criteria of instability and 90 Level 2 activations, based on mechanism of injury); five samples were collected in the pre-hospital setting, while the remainder were collected on arrival to the ED. Of these 80 patients, consent was obtained from 52; 13 were women (25%), and the overall mean age was 40 ± 17 years. The mean time between sample collection and time of injury was 2 hours (hrs) 51 minutes (min) ± 2 hrs 44 min (range 27 min – 9 hrs 22 min)**.** All enrolled subjects had received blunt mechanical trauma and a mean injury severity score (ISS) of 13 ± 11, Glasgow Coma Scale (GCS) of 13 (range 3-15), core body temperature of 37.0 ± 0.6 ° C, systolic blood pressure of 144 ± 23 mmHg, and serum lactic acid concentration of 2.2 ± 0.9 mg/dL. The hemoglobin and platelets were 13.8 ± 1.8 g/dl and 238 ± 62 x 10^9 /L, respectively. In the overall patient population, the activated partial thromboplastin times (aPTT) and international normalized ratio (INR) of study patients were within the normal reference range and did not correlate with ISS (r of 0.17 and 0.26, respectively with $p > 0.05$). Five patients had a GCS < 8 on presentation, 8 patients went directly to the operating room (OR) from the ED, and 17 patients were admitted to the Intensive Care Unit after evaluation in the ED or directly from the OR. There were two deaths during the hospitalization. One patient, 48 year old woman with an ISS of 43, developed left arm swelling due to subclavian vein thrombosis that was confirmed by venous duplex ultrasonography.

The age of the 19 outpatients used for controls was 52 ± 17 (9 men and 10 women). None of the women was on hormonal replacement therapy.

Native Calibrated Automated Thrombinogram (nCAT):

The distribution of the values of Lag Time (minutes) and thrombin Peak Height (nM) among the trauma patients and the 19 controls were broad (Figure 1) and did not differ significantly between the two groups (Table 1). Similar to the findings of Dunbar and Chandler[14] a commercial pooled control plasma (CryoCheck, Precision Biologic, Nova Scotia) had three times the Lag Time (61.4 ± 8.3 minutes) and 1/60 the Peak Height (1.3 ± 2.2 nM) of our control patients.. However, the range of peak thrombin displayed by trauma patients was nearly 30-times that compared to controls Furthermore, Lag Time and Peak Height (Figure 2) showed a significant correlation with ISS; Spearman rank correlations to Lag Time and Peak Height were r = - 0.325 (p = 0.04) and r = 0.352 (p = 0.02), respectively (Figure 2). The one patient who developed VTE had a lesser Lag Time (9 minutes) and a much greater Peak Height (298 nM) than any of the controls.

Microparticle Analysis:

Trauma patients had significantly increased total numbers of Annexin V (+) MP relative to the controls (Table 2) and with a substantially larger variance (Figure 3). The one patient who developed VTE had markedly increased numbers of Annexin V (+) MP (2,074 / µL at the time of presentation to the trauma bay). Correlation between ISS and Annexin V (+) MP was significant with r = 0.393 (p = 0.007) (Figure 4). No significant correlation was found between ISS and total platelet [CD 42a (+)] derived MP (r =0.281, p = 0.059). Interestingly, only in one patient did we detect CD 62E (+), endothelial cell-derived MP.

DISCUSSION:

In this pilot study, we have observed that the numbers of circulating thrombogenic MP and endogenous procoagulant activity in patients with blunt trauma correlate with injury severity despite normal values for

standard clotting assays. Response to injury appears to be variable between individuals as evidenced by a large variance around the regression lines. This variability is essential for exploration of an individual hypercoagulable response to trauma. Because this was a small study, we had only one patient with clinically relevant VTE. In addition, procoagulant activity in peripheral blood may continue to increase from the time of trauma and then decline as patients recover from their injuries. As we collected blood samples only at the time of patient presentation to the ED, it cannot be determined where each patient was on the injury response continuum. In our current study, serial blood draws are being obtained from trauma patient enrollees until their discharge. Our goal is to understand the importance of how changes in thrombin generation and MP distributions relative to time of injury might influence the development of VTE after trauma.

Assays that address the net effect of both pro- coagulant and anti-coagulant processes have a greater potential for detecting a hypercoagulable state than measurement of any single pathway. The CAT, which uses cell-deficient plasma, allow analysis of the composite soluble factors involved in the coagulation process, including the balance of pro- and anti-coagulant factors derived from a single individual. Compared to clotting time assays, such as prothrombin time (PT) and aPTT, which yield only 1 time point and evaluate clot formation early in the process of thrombin generation, CAT allows evaluation of total thrombin generated within a sample and is calculated as the area under the thrombin generation curve over time. Increased total thrombin generation has been associated in patients with clinical risk factors (e.g., obesity, increasing age, oral contraceptives) for VTE.[22-24]

Using the modification of the CAT that eliminates exogenous trigger (TF/PL) limits thrombin generation in response to endogenous procoagulants which, in normal subjects, is substantially less than that with a TF/PL trigger. The median Lag Time and Peak Height of our sample of patients were not different from those of 19

outpatient controls but showed a substantially greater variance, which was reflected by a positive correlation between native thrombin generation and ISS. We found that native thrombin generation from commercial reference pooled donor plasma (CryoCheck) was essentially null. This difference warrants further investigation as a publication reporting an impact of trauma on native CAT used a similar commercial product for control values.[20]

We observed significantly greater numbers of procoagulant (Annexin V+) MP in trauma patients relative to the 19 controls, and like nCAT, a high-variance correlation with ISS. MP normally found in plasma can potentially be derived from any cell type. In normal plasma, MP derived from platelets are most common (>80%), followed by MP derived from endothelial cells (<10%) and leukocytes (<10%).[25] The microparticles derived from platelets, which are the most abundant in plasma, express surface phosphatidylserine (Annexin V+) and are procoagulant in vitro. [21, 26-28] Such a thrombogenic potential of MP has been known for the past three decades, but the membrane vesicles containing phosphatidylserine (Annexin V+) are thrombogenic pharmacologically in vivo only with co-administration of a coagulant enzyme.[29 -30] Thus, endogenous MP expressing membrane phosphatidylserine may not contribute intrinsically to thrombosis but may drive thrombosis in an environment where procoagulant enzymes are generated, such as would be expected in peripheral blood of patients after major trauma.

Conclusion:

In this pilot study of patients with blunt trauma, we observed an increase in thrombogenic MP and increased thrombin generation by CAT. Future studies to characterize the profiles of MP and thrombin response over time would enhance our understanding of their role in the hypercoagulable state observed after injury. It remains to be determined if the characterization of MP and thrombin generation could find practical applications either as

diagnostic indicators or be predictive of post-injury thrombotic complications such as VTE. If so, bleeding complications related to chemoprophylaxis could be minimized by assessing the blood physiology of individual patients rather than utilizing population-based algorithms. Conversely, it may be determined that other patients require more aggressive chemoprophylaxis than is currently administered.[11]

Acknowledgment

The authors deeply thank the research coordinators (De Anna L. Haugen, Melissa Kuntz, Nancy J. Meyer, Laurnice M. Olsen and Debbie Dixon) and the Trauma Mid-level Providers (DeLayna Nitz, RN, CNP, Bethany Piotrowski, MMS, PA-C and Stacey Zimmerman, RN, CNP) for their assistance with the patient enrollment in this study. We thank our Operations Manager, John B. Osborn, MSc, for his administrative support in the funding of this study. We thank the Clinical Research Unit (CRU) of the Center for Translational Science Activities (CTSA) for their 24 hour support in sample collection. We thank the Mayo Clinic Medical Transport for their assistance in obtaining pre-hospital blood samples. We are grateful for the technical assistance received from Robert D. Litwiller and the Hematology Research Laboratory, Mayo Clinic, Rochester, MN for sample analysis.

References:

1. Brohi K, Singh J, Heron M, et al. Acute traumatic coagulopathy. J Trauma. 2003; 54:1127-1130.
2. Kauffman CR, Dwyer KM, Cews JD, et al. Usefulness of thromboelastography in assessment of trauma patient coagulation. J Trauma. 1997; 42:716-722.
3. Schreiber MA, Differding J, Thorborg P, et al. Hypercoagulability is most prevalent early after injury and in female patients. J Trauma. 2005; 58:475-481.
4. Geerts WH, Code KI, Jay RM, et al. A prospective study of venous thromboembolism after major trauma. N Engl J Med 1994; 331:1601-1606.
5. Park MS, Martini WZ, Dubick MA, et. al. Thromboelastography as a better indicator of hypercoagulable state after injury than prothrombin time or activated partial thromboplastin time. J Trauma. 2009; 67: 266 – 276.
6. Brummel KE, Paradis SG, Butenas S, Mann KG. Thrombin functions during tissue factor-induced blood coagulation. Blood 2002;100:148 - 52.
7. Regnault V, Hemker HC, Wahl D and Lecompte T. Phenotyping the haemostatic system by thrombography - potential for the estimation of thrombotic risk. Thromb Res 2004; 114: 539-545.
8. Eichinger S, Hron G, Kollars M, Kyrle PA. Prediction of recurrent venous thromboembolism by endogenous thrombin potential and D-dimer. Clin Chem 2008;54(12):2042-2048.
9. Tripodi A, Legnani C, Palareti G, Chantarangkul V, Mannucci PM. More on: high thrombin generation and the risk of recurrent thromboembolism. J Thromb Haemost 2009;7:906-907.
10. Sossdorf M, Konig V, Gummert J, Marz G, Losche W. Correlations between platelet-derived microvesicles and thrombin generation in patients with coronary artery disease. Platelets 2008;18(16):476-477.

11. Geerts WH, Bergquist D, Pineo GF, et al. Prevention of venous thromboembolism: American College of Chest Physicians Evidence-based Clinical Practice Guideline (8th edition). Chest 2008;133(6):3815 – 4535.
12. Macleod JA, Lynn M, McKenny MG, et al. Early coagulopathy predicts mortality in trauma. J Trauma. 2003;55:39-44.
13. Brohi K, Singh J, Heron M, et al. Acute traumatic coagulopathy. J Trauma. 2003;54:1127-30.
14. Kaufmann CR, Dwyer KM, Crews JD, et al. Usefulness of thrombelastography in assessment of trauma patient coagulation. J Trauma. 1997;42(4):716-722.
15. Watts DD, Trask A, Soeken K, et al. Hypothermic coagulopathy in trauma: effect of varying levels of hypothermia on enzyme speed, platelet function, and fibrinolytic activity. J Trauma. 1998;44(5):846-54.
16. Cinat ME, Wallace WC, Nastansk F, et al. Improved survival following massive transfusion in patients who have undergone trauma. Arch Surg. 1998;134:964-970.
17. Hemker HC. Recollections on thrombin generation. J Thromb Haemost 2008; 6: 219-26.
18. Hemker HC, Al Dieri R, De Smedt E, et al. Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb Haemost. 2006; 96:553 -561.
19. Owen, B.A.L, Xue, A., Heit, J.A. and Owen, W.G. Procoagulant activity, but not number, of microparticles increases with age and in individuals after a single venous thromboembolism Thr Res 2011 (127): 39-46.
20. Dunbar NM and Chandler WL. Thrombin generation in trauma patients. Transfusion. 2009; 49(12):2652 – 60.
21. Jayachandran M, Litwiller RD, Owen WG, et al. Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women. Am J Physiol Heart Circ Physiol 2008;295H931-938.

22. Eichinger S, Hron G, Bialonczyk C, et al. Overweight, obesity and recurrent venous thromboembolism. Arch Intern Med. 2008;168(15):1678-83.
23. Cimenti C, Mangge H, Haidl H, et al. Thrombin generation in severely obese children. J Thromb Haemost 2006;4:1834-1836.
24. Haidl H, Cimenti C, Leschnik B, et al. Age-dependency of thrombin generation measured by means of calibrated automated thrombography (CAT). Thromb Haemost 2006;95(5):772-775.
25. Bereckmans RJ, Neiuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost. 2001;85:639-646.
26. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood. 1997; 89:1121 -1132.
27. Morel O, Toti F, Hugel B, et al. Procoagulant microparticles: disrupting the vascular homeostasis equation? Arterioscler Thromb Vasc Biol. 26(12):2594-604, 2006 Dec.
28. Bereckmans RJ, Neiuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost. 2001;85:639-646.
29. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood. 1997; 89:1121 -1132.
30. Barton PG, Yin ET, Wessler S. Reactions of activated factor X-phosphatide mixtures in vitro and in vivo. J Lipid Res. 1970;11(2):87-95.

Figure Legends:

Figure 1: Peak Height distributions of native thrombin generation. The top of each box defines the 75th percentile, while the bottom of the box the 25th percentile and the line through the box indicates the median. The top whiskers define the 95th percentiles and the bottom whiskers define the 5th percentiles of the data. Each data point is represented by a symbol.

Figure 2: Correlations between Injury Severity Score and Peak Height. The data were fit using a linear regression model.

Figure 3: Distribution of Annexin V Positive Microparticles counts. Box and whisker variables as defined in figure 2. Note that there are 7 trauma patients that fall outside the 95th percentile.

Figure 4: Correlation of Injury Severity Score with Annexin V Positive Microparticle counts. Linear regression analysis as described in Figure 2.

Table 1: Native Calibrated Thrombinogram (nCAT) of Plasma

	PATIENTS (N = 40)	CONTROL (N=19)	P VALUE
Native Lag Time (min)	26.9 ± 14.9	22.6 ± 7.3*	0.615
Native Peak (nM)	77.0 ± 62.0	76.9 ± 40.4*	0.372

*From Mayo Clinic outpatients with no prior history of thrombosis.

Table 2: Microparticle with Platelet - Derived Annexin V Positive (Annexin V +) Microparticles (MP) in row 1; All Annexin V + MP in row 2

	PATIENTS (N = 47)	CONTROL (N = 19)	P VALUE
Platelet Derived Annexin V + MP	328 ± 1418	151 ± 145	<0.001
Total Annexin V + MP	541 ± 1981	155 ± 148	<0.001

Figure 1

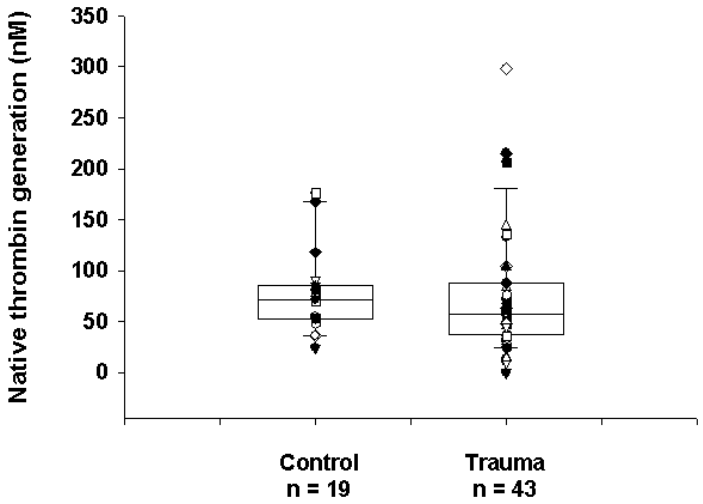


Figure 2

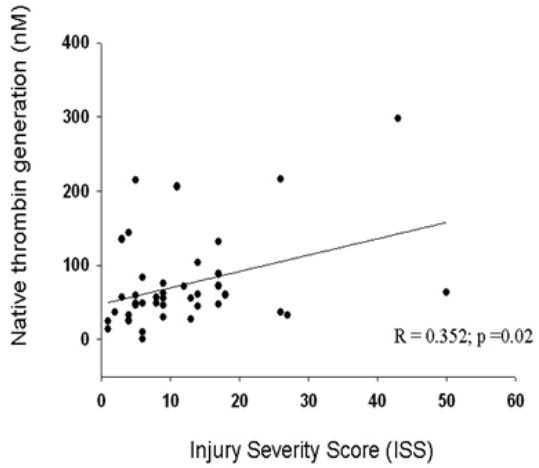


Figure 3

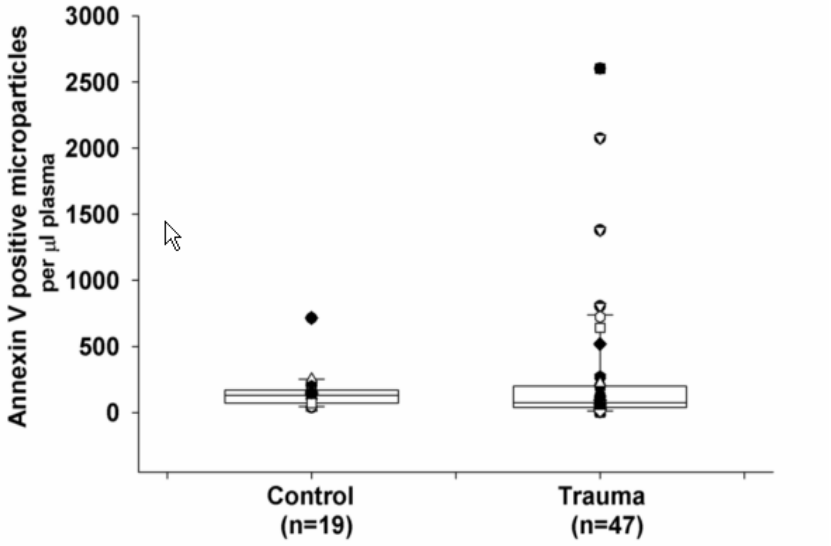
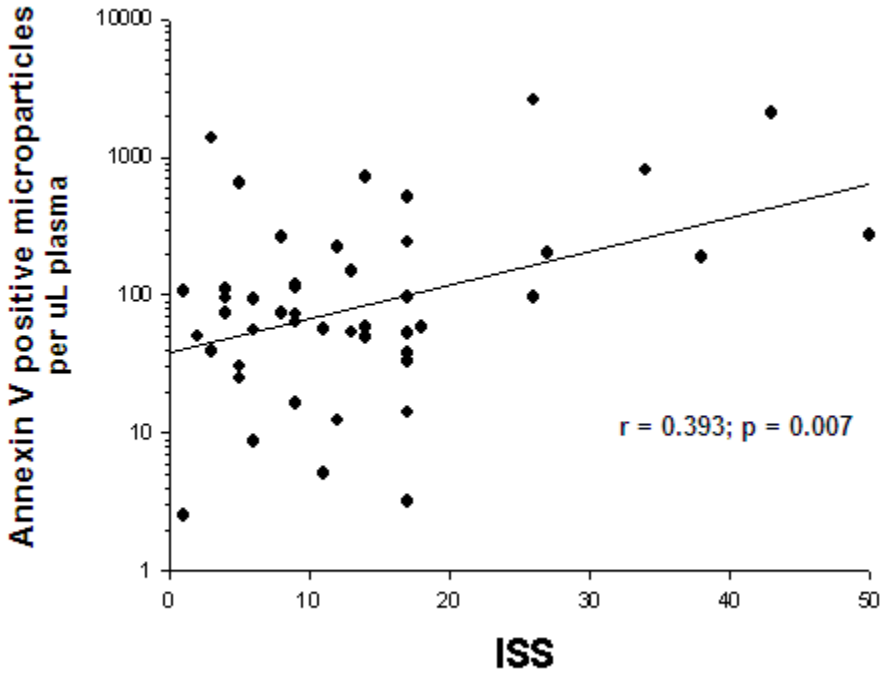


Figure 4



Supporting Data: None